A subset of high Gleason grade prostate carcinomas contain a large burden of prostate cancer syndecan-1 positive stromal cells

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Disclosure Statement

The authors declare that there are no conflicts of interest to report.

Abstract

Background

There is a pressing need to identify prognostic and predictive biomarkers for prostate cancer to aid treatment decisions in both early and advanced disease settings. Syndecan-1, a heparan sulfate proteoglycan, has been previously identified as a potential prognostic biomarker by multiple studies at the tissue and serum level. However, other studies have questioned its utility.

Methods

Anti-Syndecan-1 immunohistochemistry was carried out on 157 prostate tissue samples (including cancerous, adjacent normal tissue and non-diseased prostate) from three independent cohorts of patients. A population of Syndecan-1 positive stromal cells was identified and the number and morphological parameters of these cells quantified. The identity of the Syndecan-1-positive stromal cells was assessed by multiplex immunofluorescence using a range of common cell lineage markers. Finally, the burden of Syndecan-1 positive stromal cells was tested for association with clinical parameters.

Results

We identified a previously unreported cell type which is marked by Syndecan-1 expression and is found in the stroma of prostate tumors and adjacent normal tissue but not in non-diseased prostate. We call these cells Prostate Cancer Syndecan-1 Positive (PCSP) cells. Immunofluorescence analysis revealed that the PCSP cell population did not co-stain with markers of common prostate epithelial, stromal or immune cell populations. However, morphological analysis revealed that PCSP cells are often elongated and displayed prominent lamellipodia, suggesting they are an unidentified migratory cell population.

Analysis of clinical parameters showed that PCSP cells were found with a frequency of 20-35% of all tumors evaluated, but were not present in non-diseased normal tissue. Interestingly, a subset of primary Gleason 5 prostate tumors had a high burden of PCSP cells.

Conclusions

The current study identifies PCSP cells as a novel, potentially migratory cell type, which is marked by Syndecan-1 expression and is found in the stroma of prostate carcinomas, adjacent normal tissue, but not in non-diseased prostate. A subset of poor prognosis high Gleason grade 5 tumors had a particularly high PCSP cell burden, suggesting an association between this unidentified cell type and tumor aggressiveness.

Key Words:

Syndecan-1; SDC1; CD138; prostate cancer; immunohistochemistry; stromal cells

Introduction

Prostate cancer accounts for almost a quarter of all cancers diagnosed in European men, with a large increase in incidence over the past 20 years due to increased detection through PSA testing. Radical treatments such as surgery or radiotherapy have considerable morbidity and there is increasing enthusiasm for a period of active surveillance in patients with low volume, low-intermediate risk disease. However, we currently do not have any useful biomarkers beyond Gleason grade to help inform which patients would be better suited to surveillance. Likewise, in the case of metastatic prostate cancer, there is a lack of useful biomarkers to help direct the choice and sequencing of drug treatments such as chemotherapy, abiraterone and enzalutamide. One potential biomarker that has been investigated is the heparan sulfate proteoglycan Syndecan-1.

The expression of Syndecan-1 has been linked to prognosis and treatment response in a large range of cancer types, including hematolymphoid malignancies and solid tumors (1-6), but in prostate cancer the role of Syndecan-1 in prognosis is controversial. It is expressed in the basal epithelial layer of normal prostate glands, and this expression is often lost in prostate carcinoma cells (7-12). However, there are reports that hormone-refractory tumors (9) and tumors treated with neo-adjuvant hormone therapy (8) have increased Syndecan-1 expression, suggesting a relationship between expression and androgen independence. There is also evidence that expression of Syndecan-1 is associated with high Gleason grade disease and/or early disease recurrence in prostate cancer patients (7-11). Interestingly, an alteration in protein localization, from membranous to cytoplasmic, was found to be associated with a more rapid biochemical recurrence (13). This indicates that alterations in Syndecan-1 expression and localization could be prognostically important and change throughout the course of the disease. Conversely, others have found that Syndecan-1 expression is not associated with recurrence-free survival (9,14) or Gleason grade in prostate carcinoma (9), and even concluded that lower levels of Syndecan-1 expression were associated with a high Gleason grade (1). Therefore, the potential utility of Syndecan-1 as a biomarker for prostate cancer is far from clear.

The function that Syndecan-1 plays in normal and cancerous tissues is also complicated. Syndecan-1 protein has been linked to a wide range of cellular processes, acting as an extracellular matrix receptor as well as participating in growth factor binding through the heparan sulfate chains on its extracellular domain (Reviewed in (1)). The extracellular domains of Syndecan-1 can also be shed through cleavage by matrix metalloproteinases, creating a soluble form which may have pro-angiogenic roles in tumors, for example in myeloma (1), and this shed ectodomain in itself may possess prognostic

utility (12). There is also evidence that Syndecan-1 signaling is involved in maintaining the tumor-initiating cell/cancer stem cell population in breast carcinoma (15,16) and prostate carcinoma (17) and can regulate the expression of several miRNAs that suppress expression of stem cell markers such as SOX-2, OCT4 and Nanog (11). Suppression of Syndecan-1 gene expression also reduces amplification of the putative tumor-initiating cell population following treatment with docetaxel (17), suggesting that it could exert effects of relevance to prostate cancer patients undergoing therapy. Taken together, there is accumulating evidence that Syndecan-1 is an important multifunctional molecule that shows altered expression in prostate cancer, but its potential role as a biomarker for prostate cancer remains controversial.

Given the interesting functional roles played by Syndecan-1 and the conflicting evidence surrounding its possible utility as a biomarker, we investigated Syndecan-1 expression in three independent cohorts of prostate carcinoma samples. We identified a previously unreported cell type which is marked by Syndecan-1 expression and is found scattered in the stroma of a third of prostate carcinomas and in their adjacent normal tissue, but not in normal tissue. We call these cells Prostate Cancer Syndecan-1 Positive (PCSP) cells. Common markers for immune cells, cancer epithelial cells and stromal cells failed to identify the cell type, but further analysis showed that PCSP cells are elongated and have prominent lamellipodia, suggesting they are a migratory cell type. Finally, comparison of the relationship between PCSP cell burden and clinical features showed that a subset of poor prognosis high grade tumors have a particularly high burden of these cells.

Materials and Methods

Patients

The study was conducted following local ethics approval (REC reference: 13/WS/0153; IRAS project ID: 112241). Data was obtained from three separate cohorts of patients with associated formalin-fixed, paraffin-embedded (FFPE) prostate material available. The total number of cancer cases in the study was 157. The Bath cohort was used for analysis of cell types by co-staining, and consisted of 6 prostate cancer patients with confirmed Syndecan-1+ stromal cells as assessed by anti-Syndecan-1 IHC. In addition, one block of adjacent normal prostate tissue was used as a positive control for Syndecan-1 staining. These samples were obtained retrospectively from patients treated for prostate cancer at the Royal United Hospital, Bath, United Kingdom, between 1997 and 2008. The other two cohorts, consisting of 151 cancer cases in total, were used for anti-Syndecan-1 immunohistochemistry and were obtained from US Biomax (Rockville, MD, USA). The first cohort (PR1921) consisted of 80 cases of prostate adenocarcinoma with 8 cases of adjacent normal prostate tissue and 8 cases of histologically normal prostate tissue, with duplicate 1mm diameter cores per cancer case for a total of 196 cores. The second cohort (PR803b) consisted of 71 cases of prostate adenocarcinoma, 2 cases of prostate leiomyosarcoma, 1 case of benign prostate hyperplasia and 6 cores of histologically normal prostate, with a single 1.5mm core per case. Each cohort was contained in a single tissue microarray block. A summary of the patient characteristics of tissue microarray cohorts is given in Table I.

Immunohistochemistry

For immunohistochemistry, 5um thick sections of FFPE prostate tissue or tissue microarrays were used. Cohort 1 and cohort 2 were stained in separate experiments. Briefly, sections were baked for 30 minutes at 60°C before being deparaffinized in Histoclear (National Diagnostics, Hessle, UK) and rehydrated through a graded ethanol series of decreasing ethanol concentration. Sections were washed with PBS-tween (0.05%), permeablized with 0.5% triton X-100 in PBS, subjected to heat-induced epitope retrieval in 10mM Citrate buffer with 0.05% Tween 20 at pH 6.0 for 30 minutes at 90°C, and allowed to cool to room temperature for 20 minutes. Endogenous peroxidases were blocked with Dako Peroxidase Block, following the manufacturer's instructions. After blocking for 30 minutes in 10% normal goat serum and 0.5% BSA in PBS, samples were incubated with primary antibodies (Table II) diluted in Dako Antibody Diluent (Dako, Ely, UK) overnight at 4°C. The next day, samples were washed and bound antibodies were visualized using the EnVision+ Kit (K400611-2 and K401011-2, Dako, Ely, UK) following the manufacturer's instructions with DAB exposure for 5 minutes. Nuclei were counterstained with Gill's Hematoxylin (H-3401,Vector Laboratories, Peterborough, UK) and mounted in DPX (Sigma-Aldrich, Dorset, UK). Stained tissues were viewed under a Nikon Eclipse E800 microscope with brightfield illumination and photographed with a Nikon Digital Sight DS-U1 CCD Digital Camera. Negative controls were IHC-stained sections of prostate carcinoma tissue with no primary antibody added, and showed no positive staining (data not shown).

Anti-Syndecan-1 Antibodies

Three distinct Syndecan-1 antibodies were used (Table II). The three antibodies were selected as they have been previously shown to successfully detect Syndecan-1. The first mouse monoclonal anti-Syndecan-1 antibody, (Novus Biologicals: clone B-A38: Cat NB100-

64980), later referred to as mo anti-SDC1 #1, has been shown to robustly mark a CD45-expressing plasma cell population by flow cytometry (18), a cell population which expresses Syndecan-1 (19). The second mouse monoclonal (Dako; clone MI15: Cat M7228) has been shown to recognize the ectodomain of Syndecan-1 (20). Antibody blocking experiments show that it partially shares the epitope of B-B4, which occurs at residues 90-93 of the mature Syndecan-1 protein (21). The rabbit polyclonal anti-Syndecan-1 antibody (Santa Cruz Biotechnology: SC-5632) was generated by immunization with amino acids 82-256 of human Syndecan-1, corresponding to the human Syndecan-1 ectodomain, and successfully immunoprecipitated the ectodomain (22), showing that the antibody can detect the cleaved ectodomain of the protein. The rabbit antibody is also known to detect Syndecan-1 positive plasma cell populations by immunohistochemistry (23).

Scoring

For assessment of IHC staining on the two tissue microarrays, whole cores were examined under a 10x objective to determine the presence of Syndecan-1 staining in prostate epithelium. Cores were scored as either positive or negative for Syndecan-1 epithelial reactivity based on the observed staining patterns across the core.

For quantification of PCSP cell burden, fields of view of all PCSP cells in every core were manually acquired under a 20x objective. Counting and measuring of the resulting images was performed using ImageJ v2.0.0. Each PCSP cell was identified manually and the following size and shape descriptors were measured: area, circularity, aspect ratio, roundness and solidity. Mean values were taken for each measure within each cohort as a whole, and for each patient.

Data Analysis

To analyze the distribution of Syndecan-1+ cell burden amongst different patient groups in both TMA cohorts, we classified patients into Syndecan-1 stromal cell positive and negative categories based on whether Syndecan-1+ stromal cells were present or absent in the core. In the case of PR1921, where duplicate cores were scored, patients were classified as positive if at least one core contained Syndecan-1+ stromal cells. The distributions of Syndecan-1+ and Syndecan-1- patients amongst different levels of clinical variables were tested with the chi-squared test performed in R version 3.2.0 with default parameters. Clinical variables assessed were: age; stage; Gleason grade; T, N and M stages; Presence of Syndecan-1 staining in the glandular compartment was also scored as positive or negative, and tested for association as with clinical variables. A p-value of less than 0.05 was considered statistically significant. Graphs were generated in R version 3.2.0.

Immunofluorescence

For immunofluorescence co-staining of Syndecan-1 with other markers, double-labelling was performed on FFPE prostatectomies, biopsies and TURP chips with confirmed PCSP cells as determined by prior IHC experiments. 5um thick sections were deparaffinized, rehydrated, permeablized and subjected to antigen retrieval as for immunohistochemistry. Omitting the peroxidase blocking step, samples were blocked with the same blocking buffer and incubated with a mixture of two primary antibodies raised in different species, overnight at 4°C, as before. Cell type markers and either the rabbit polyclonal or the first mouse monoclonal (mo anti-SDC1 #1) anti-Syndecan-1 antibodies were chosen, depending on the host in which the marker antibody was raised. The antibodies used in this study are described in Table II. The following day, samples were washed and incubated in a secondary antibody cocktail containing goat anti-mouse alexafluor-488 (A-11001, Invitrogen) and goat anti-rabbit alexafluor-568 (A-11008, Invitrogen), each at 1:200 dilutions in Dako Antibody

Diluent, for 30 minutes. The specimens were then incubated with a solution of 0.1% (w/v) Sudan Black B (199664, Sigma-Aldrich, Dorset, UK) in 70% ethanol for 10 minutes to quench endogenous tissue autofluorescence, as adapted from (24). Mounting was achieved in Mowiol-4-88 (Millipore) with 600nM DAPI (D9542, Sigma-Aldrich) added. Slides were allowed to set overnight in the dark at room temperature before imaging on a ZEISS LSM510 META confocal laser scanning microscope. Negative controls were IF-stained sections of prostate carcinoma tissue with no primary antibody added, and never showed positive staining (data not shown).

To score Syndecan-1+ stromal cells for their colocalization with test markers, images from each experimental run were pooled, and each Syndecan-1+ cell was manually scored for positivity of the co-stained marker. The percentage of marker-positive cells was calculated as the percentage of Syndecan-1+/Marker+ cells relative to the total number of Syndecan-1+ cells in an experimental run.

Results

A population of Syndecan-1 positive cells was found in the stromal compartment of a subset of prostate tumors

Syndecan-1 expression was analyzed using IHC and IF in samples from three independent cohorts of patients (See Materials and Methods). Expression was found in the cell membrane and cytoplasm of the prostate epithelium basal, but not luminal, cell layer in histologically normal tissue (Figure 1A, Left Panel). In prostate carcinoma tissues there was either a loss of epithelial staining or a widespread expression of the proteoglycan throughout the cytoplasm and/or cell membranes of prostate tumor epithelium (Figure 1A, Middle and right Panel). These patterns are consistent with previous studies

(3,7,10,12,13,25) and provide positive (basal prostate epithelium) and negative (luminal prostate epithelium) control cells for the IHC staining. Interestingly, in addition to these staining patterns, a population of scattered Syndecan-1 positive cells was found in the stroma in a subset of cancer cases (Figure 1A). These scattered cells were present in a subset of tumor samples from each of the three cohorts, either proximal or distal to an epithelial region. A range of examples, including samples from all three cohorts, are shown in Figure 1B. The staining was found to be localized to the membranes and/or cytoplasmic regions of the Syndecan-1 positive cells, and the cells often appeared in close proximity to areas of apparent inflammation, characterized by the presence of stromal cells with round, compact and hyperchromatic properties suggestive of an immune cell phenotype (Figure 1B, Adjacent to stained cells in the inserts and near arrowheads. The bottom left panel insert shows a clear example).

To confirm that the staining was due to the presence of Syndecan-1, and not cross reactivity from the mouse monoclonal antibody (Clone B-A38), two additional anti-Syndecan-1 antibodies were used. A rabbit polyclonal antibody and a second mouse monoclonal (Clone MI15) were used. Staining of sequential sections of normal prostate and tonsil by IHC showed that all three antibodies stained the same basal, but not luminal, epithelial cell populations (Figure 2A and B). This specific pattern of expression provides positive and negative controls for the three antibodies and, combined with validation of the antibodies carried out in previous publications (see materials and methods), argues that they can be used to reliably identify Syndecan-1 positive cells. The two additional anti-Syndecan-1 antibodies (rabbit polyclonal and second mouse monoclonal), like the original mouse monoclonal antibody, also detected scattered Syndecan-1 positive cells in the stroma of prostate tumors (Figure 2C).

A final experiment to validate the anti-syndecan-1 antibodies was carried out using IF staining of normal and prostate carcinoma tissues. This made it possible to establish if the different antibodies were staining the same populations at a single cell level. IF staining using the mouse monoclonal #1 and the rabbit polyclonal antibody showed colocalisation of Syndecan-1 staining in individual basal, but not luminal, epithelial cells of adjacent normal prostate (Figure 3, upper panels). Importantly, colocalization of Syndecan-1 staining was also seen in the scattered tumor stromal cells (Figure 3, lower panels arrows and inserts), confirming that the two antibodies stain the same population of scattered stromal cells.

In summary, the use of three independent antibodies showed that a subset of prostate cancer samples, from three cohorts of patients, contained a population of scattered Synecan-1 positive cells, which to our knowledge have not been previously described. We call these cells Prostate Cancer Syndecan-1 Positive (PCSP) cells.

PCSP cells do not express common epithelial, mesenchymal or immune cell markers

To begin to characterize the PCSP cell population, we used double-IF labelling with Syndecan-1 and a range of markers commonly used to distinguish key cell types found in prostate tumor samples from the Bath cohort. Given that Syndecan-1 is expressed in normal and neoplastic plasma cells (19), we began by assessing the expression of hematolymphoid lineage markers in PCSP cells. Fluorescent co-stains with Syndecan-1 and a panel of antibodies raised against immune cell lineage markers: CD45, which is expressed in the majority of hematolymphoid lineages with the exception of erythrocytes and some macrophages, and known to be expressed in Syndecan-1+ bone marrow plasma cells (18); CD19, which is expressed on immature and memory B-cells (26); CD27, which is expressed in

plasma cells, memory B-cells (26) and T-cells (27); and CD73, an ecto-5'-nucleotidase expressed on subsets of T and B lymphocytes (28), as well mesenchymal stem cells which have been previously shown to infiltrate prostate tumors (29). To confirm the antibodies for these markers were performing as expected they were used to stain positive control tonsil or placenta tissues (Supplementary Figure 1). In each case the staining was as expected.

In prostate tumors, the majority of PCSP cells did not co-stain for any of the three immune cell markers or the mesenchymal stem cell marker (Figure 4A+B). Interestingly, despite the lack of colocalization, PCSP cells were often found in close proximity to CD45-positive immune cells (Figure 4A: See inserts in the top panel). This was consistent with IHC data showing that Syndecan-1 positive cells were found in close proximity to cells whose nuclear morphology was consistent with an immune cell phenotype (Figure 1B, Adjacent to stained cells in the inserts and near arrowheads. The bottom left panel insert shows a clear example).

Given that Syndecan-1 is expressed in the basal cells of normal prostate, another possible origin for the PCSP population was prostate epithelial cells, so co-staining was carried out for Syndecan-1 and three markers of epithelial cell fate: pan-Cytokeratin, which stains multiple human cytokeratins expressed in epithelial tissues including both basal and luminal prostate epithelial cell layers (Figure 5A); E-Cadherin, which is normally expressed throughout the normal prostate epithelium and in most prostate tumors (30); and Prostate-Specific Antigen (PSA), a secretory marker of luminal prostate epithelium, also expressed in prostate tumors. Quantification of the IF images showed none of these markers had significant co-staining with Syndecan-1 in PCSP cells (Figure 5B). There was also no apparent overlap in the IHC staining of the stromal cells in consecutive tissue sections for Syndecan-1 and the basal cell markers p63 and Cytokeratin-5 (Figure 5C, lower panels). This was clearly

distinct from the situation in the basal cells of adjacent normal tissue, where the markers do overlap with Syndecan-1 staining (Figure 5C, upper panels). Thus, despite the overlap in normal epithelium, PCSP cells did not have staining patterns consistent with them being a recognized prostate epithelial cell population, whether basal or luminal in origin.

To examine if the PCSP cells were stromal, mesenchymal or had undergone an epithelial-mesenchymal transition and lost expression of epithelial markers, a panel of stains for mesenchymal and stromal cell types was examined. The mesenchymal cell markers Vimentin and N-Cadherin did not show significant co-staining with PCSP cells (Figure 6A+B). Finally, the neural marker S100 and the endothelial marker CD31 were examined (Figure 6A), neither of which showed significant overlap (Figure 6B). Therefore, the marker analysis carried out did not allow the allocation of PCSP cells to an established cell population, and they remained an undefined cell type.

PCSP cells are elongated and have prominent lamellipodia-like structures

In order to characterize this unidentified cell type further, the shape of PCSP stromal cells was analyzed using IHC stained tissue microarrays from two independent cohorts of prostate cancer patients. The circularity and aspect ratios of the PCSP cells were quantified and on average PCSP cells were found to be elongated rather than rounded in shape (Table III). When morphological data from individual cells of both TMA cohorts were pooled, a tail of elongated cells with high aspect ratio and low circularity was apparent (Figure 7A). To further characterize this subpopulation of elongated cells, confocal laser scanning microscopy was used to carry out high resolution three dimensional imaging of the PCSP cells. They were found to have striking lamellipodia-like protrusions (Figure 7B, arrows) and

polarized cell bodies and nuclei (Figure 7B, arrowheads). The elongated shape and prominent protrusions support the hypothesis that PCSP cells may be a migratory cell type.

A subset of Gleason grade 5 tumors had a particularly high PCSP cell burden

Using the clinical metadata available for both prostate tissue microarray cohorts, we wished to determine if there was a relationship between PCSP cell burden and clinicopathological features. These included age, Gleason grade and TNM stages. In the first cohort, cores of histologically normal tissue adjacent to prostate cancer were also included, as well as non-diseased normal prostate control cores. Patient characteristics for both cohorts are summarized in Table I, and have similar patient distributions for the clinical variables.

PCSP cells were found in 28 of 80 cases (35%) of patients with prostate cancer in cohort 1, but they were not observed in any of the 8 control cases of non-diseased patient tissue (Figure 8A). PCSP cells were found in 4 of 8 cases of normal patient tissue that was adjacent to carcinoma (Figure 8A). In cohort 2, PCSP cells were found in 15 of 71 prostate cancer cases (21.1%). In both cohorts, a subset of Gleason grade five tumors had a particularly high burden of PCSP cells (Figure 8B+C). Among cohort 2, but not cohort 1, there was also a statistically significant association between PCSP cell burden and higher primary Gleason grade ($p<0.05$). In summary, analysis of PCSPs in the tissue microarrays showed that PCSP cells were not observed in normal prostate tissue, but were found in adjacent normal and in roughly 1/3 of prostate carcinoma tissues, with a subset of higher grade tumors having a particularly high burden.

Discussion

This study identifies PCSP cells as a previously unreported Syndecan-1-positive cell population found in the stroma of prostate adenocarcinoma, in adjacent normal tissue, but not in non-diseased prostate. These PCSP cells do not express lineage markers of common epithelial, stromal or immune cell lineages, but do display elongated morphologies with lamellipodia-like structures consistent with a migrating cell type. PCSP cells appear in high burdens in a subset of primary Gleason grade 5 poor prognosis tumors.

The existence of PCSP cells was confirmed using three separate antibodies recognizing Syndecan-1; in both immunohistochemistry and immunofluorescence, using samples from three different patient cohorts. However, several previous studies have specifically reported the absence of stromal Syndecan-1 staining in prostate tumors (7,10,12). It is possible that the focal nature of this cell type – and their presence only in a subset of tumors - has led to them being previously overlooked.

Immune cells are commonly found in prostate tumors and Syndecan-1 is a marker of plasma cell differentiation, being expressed at the later stages of the transition from plasmablast into a mature plasma cell (31). However, we were unable to identify an immune cell marker profile in PCSP cells. The fact that plasma cells tend to express both Syndecan-1 and the pan-leukocyte marker CD45 (18), whilst PCSP cells are CD45-, would suggest that PCSP cells are either an atypical plasma cell or a non-plasma cell population. Tumor-associated macrophages - a component commonly associated with inflammation in tumors – also express CD45, being a leukocyte population, and that makes it unlikely that PCSP cells represent a subset of that population. However, given the enormous diversity in the

immune cell repertoire, it is still possible that PCSP cells represent an immune cell population.

Co-staining with epithelial markers again failed to mark a significant portion of the PCSP cell population, despite the overlap seen in adjacent normal epithelium. There remains the possibility that PCSP cells could have undergone dedifferentiation, as they do not possess an epithelial identity consistent with tumor cells. Epithelial-Mesenchymal Transition (EMT) is an unlikely explanation for this observation, as PCSP cells also lacked mesenchymal markers such as Vimentin and N-Cadherin (Figure 5A+B). Another possibility for the lack of differentiation markers is a stem-like status, either derived from a resident prostate cell population or from a mobilized stem cell niche such as Mesenchymal Stem Cells (32) or cancer stem cells, although co-staining with a Mesenchymal Stem Cell marker (CD73) did not show any colocalisation with PCSP cells. There is evidence that Syndecan-1 signaling is involved in maintaining the tumor-initiating cell/cancer stem cell population in the prostate carcinoma cell line PC-3, where its expression marks a population of CD133+/CD44+ stem-like holoclones (17), so a migratory dedifferentiated/stem like epithelial cell identity remains a possibility. It is also possible that Syndecan-1 might stain more than one population of cells in the stroma that overlap with different markers. We feel this is a less likely than a single population of PCSP cells, but a key question for future work is to identify the markers expressed by these cells to establish their identity and whether they represent more than one population of cells.

Analysis of PCSP morphology suggests a migratory cell type, something that is supported by the finding that histologically normal tissue adjacent to a carcinoma can harbor PCSP cells. A migratory PCSP cell population could be either recruited from other tissues, consistent with an immune cell type; a hypothesis which is supported by the close

proximity of other immune cells to the PCSP cells. However, PCSP cells might also be migrating out from the prostate tumor and recruiting or being detected by immune cells, which would be more supportive of a dedifferentiated/stem like epithelial population.

In addition to their characterization, it is important to determine the clinical relevance of PCSP cells. We observed that there is a trend towards increased burden in the primary Gleason grade 5 tumors, suggesting that PCSP burden might be related to outcome given that assignment of primary Gleason grade 5 is a poor prognostic factor. While cohort 2 showed a significant association between primary Gleason grade and the presence of a PCSP cell burden ($p<0.05$), cohort 1 did not reach significance ($p=0.706$). This could be due to differences in sampling strategy between the two cohorts because of different core numbers and diameters, with cohort 1 having duplicate 1mm cores and cohort 2 having single 1.5mm cores. This could lead to differences in successful detection of Syndecan-1+ cells in tumors given their scattered nature. Further investigation is warranted to determine the relationship between PCSP burden and grade, and prognostic utility of PCSP cells in prostate carcinoma, as well as any potential for predicting which cancers may respond better to upfront systemic cytotoxic or hormone treatments, and which may benefit from active surveillance.

Conclusions

This report is, we believe, the first to identify PCSP cells, an apparently migratory Syndecan-1+ cell population found in the stroma of prostate tumors and adjacent normal tissue, but not in non-diseased prostate. In addition, we demonstrate an increased burden of this cell type in primary poor prognosis Gleason pattern 5 tumors. These findings provide

a foundation for further characterization of PCSP cells, their clinical relevance and their roles in prostate tumors and the adjacent normal tissue.

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Figure Legends

Figure 1. Scattered Syndecan-1 positive cells are found in the stroma of prostate carcinoma tissue. (A) Expression of Syndecan-1 in adjacent normal and prostate adenocarcinoma tissue. Syndecan-1 is expressed in the basal cells of adjacent normal tissue. In prostate carcinoma Syncecan-1 expression is lost or found to be broadly expressed in the

epithelium. In addition, in a subset of prostate cancer cases Syndecan-1 positive cells were found scattered in the stroma (shown in the insets and by arrows), but not in adjacent normal prostate tissue. We call these cells Prostate Cancer Syndecan-1 Positive (PCSP) cells and they could be found in the contexts of both epithelial Syndecan-1 alterations in tumors, including expression across all epithelial cells, and where Syndecan-1 expression is lost in the epithelium. (B) Examples of PCSP cells detected by anti-Syndecan-1 IHC. PCSP cells were located in the stroma and tend to appear near regions that appear to contain immune cells with small, round, hyperchromatic nuclei. Images are from 3 Independent patient cohorts. Nuclei are counterstained with hematoxylin (blue) in IHC, or with DAPI (blue) in IF. IHC scale bars – 100um; IF scale bars – 50um. SDC1 – Syndecan-1.

Figure 2. Three distinct anti-Syndecan-1 antibodies show the expected patterns of Syndecan-1 expression in control prostate and tonsil tissue, and stain scattered stromal PCSP cells. (A) Staining of adjacent tissue sections of a representative adjacent normal prostate and tonsil positive controls with two different mouse monoclonal antibodies recognizing Syndecan-1. Both antibodies stained basal cells in adjacent normal prostate epithelium and epithelium in tonsil as expected. (B) Staining of adjacent tissue sections of representative adjacent normal prostate and tonsil positive controls with a mouse monoclonal and rabbit polyclonal antibodies that both recognize Syndecan-1. These antibodies also stained basal cells in adjacent normal prostate epithelium and epithelium in tonsil as expected. (C) Three anti-Syndecan-1 antibodies recognise similar stromally-located PCSP cells in both prostate tumors and in adjacent normal prostate tissue. Example cells are highlighted in the magnified insets and with the arrows. Nuclei are counterstained with hematoxylin (blue) for IHC. Scale bars – 100um with inserts at 3x zoom; SDC1 – Syndecan-1; mo – mouse; rb – rabbit.

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Figure 7. PCSP cells are elongated and have prominent lamellipodia-like structures. (A) Pooled single cell measurement data of PCSP cells from both tissue microarray cohorts. A continuum between a more rounded cell morphology (circularity and aspect ratio close to 1) and a tail of cells assuming a more elongated cell shape with lower circularity and higher aspect ratio was observed. (B) Sequential slices through a z-stack of images of PCSP cells in a prostate tumor tissue section. PCSP cells had an elongated cell morphology, with polarized nuclei and lamellipodia-like protrusions. Optical sections are 1um apart. Blue: DAPI; Green: Syndecan-1. Scale bar: 10um. SDC1 – Syndecan-1.

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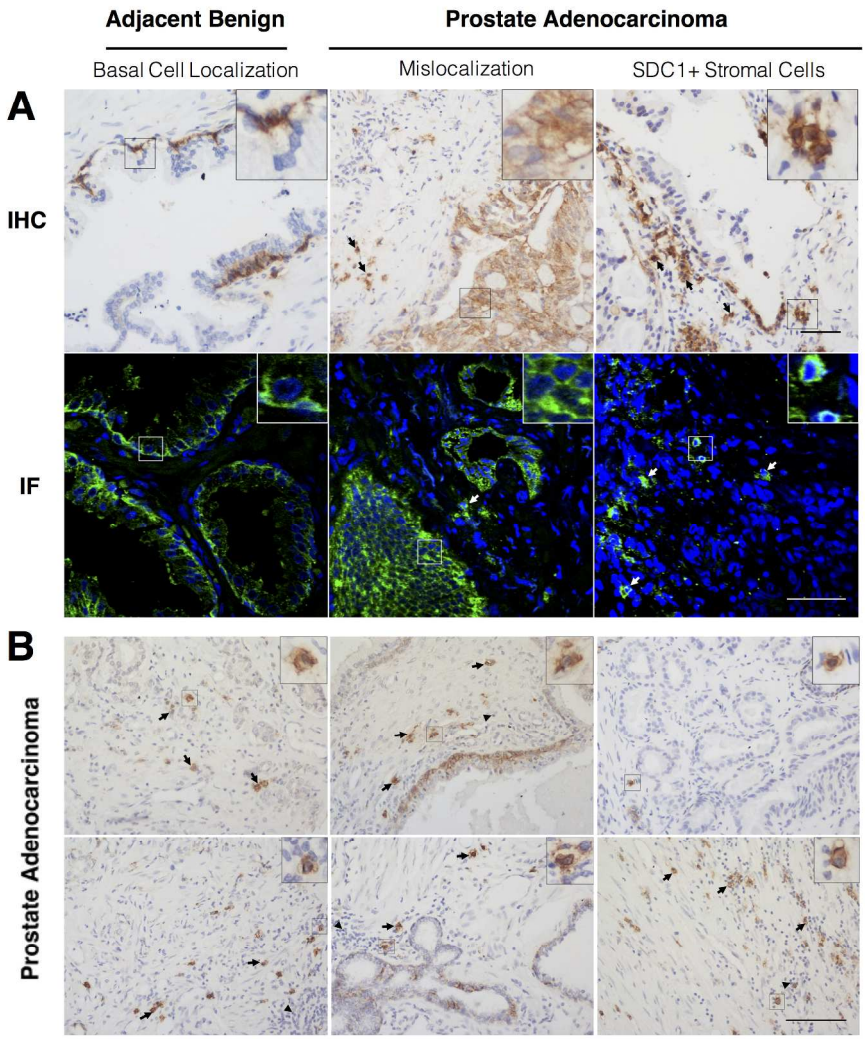

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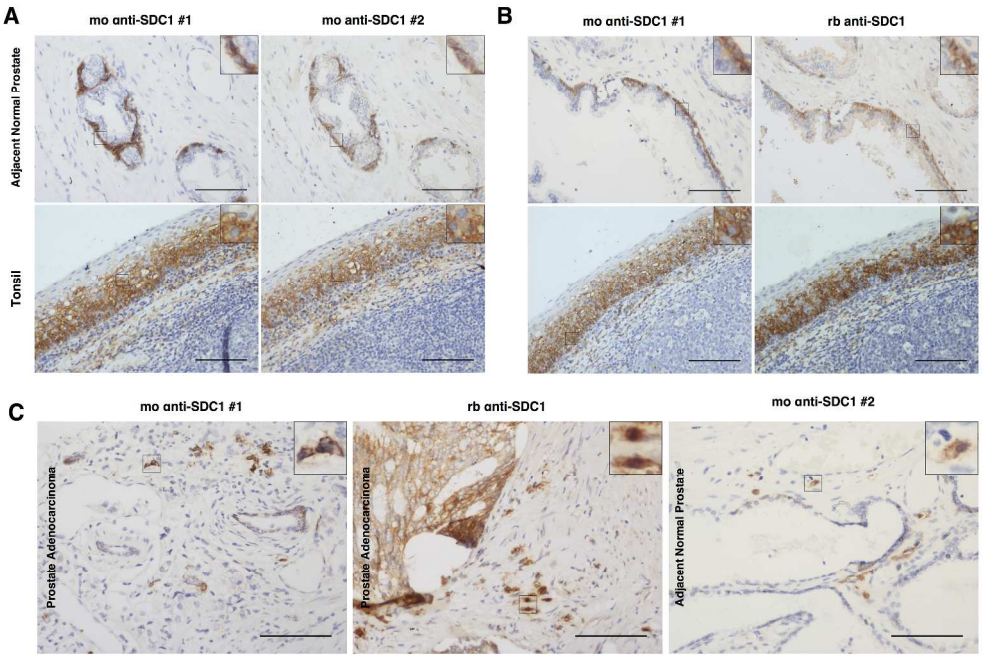

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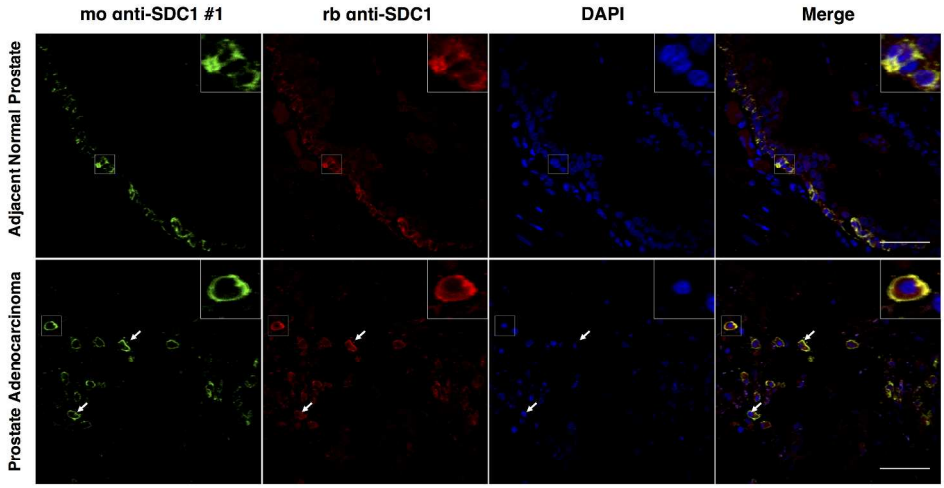

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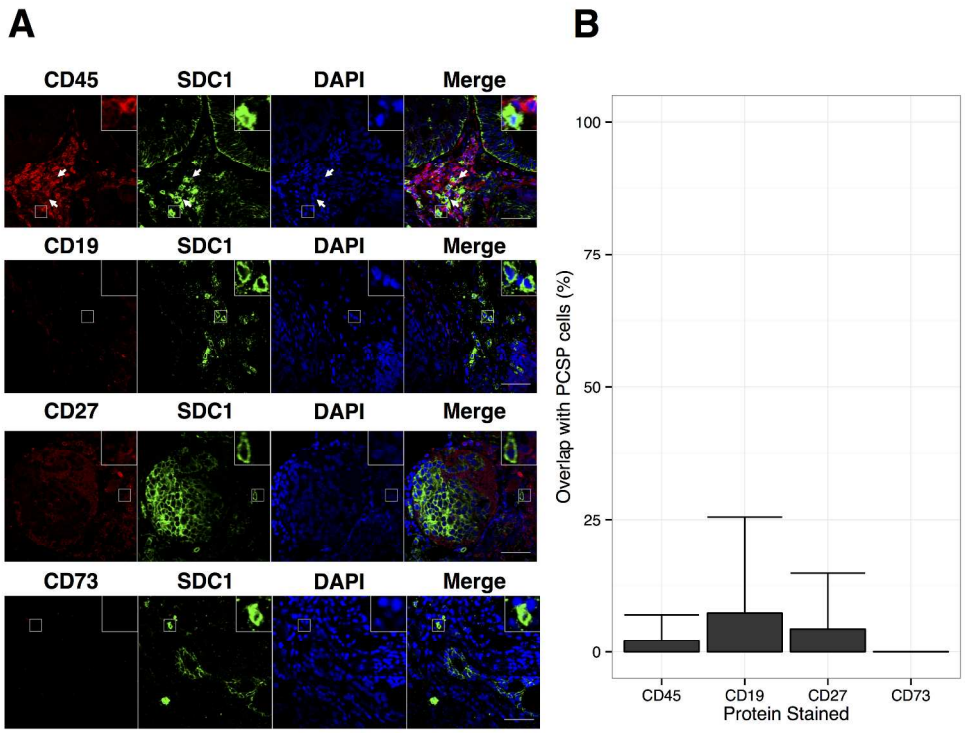

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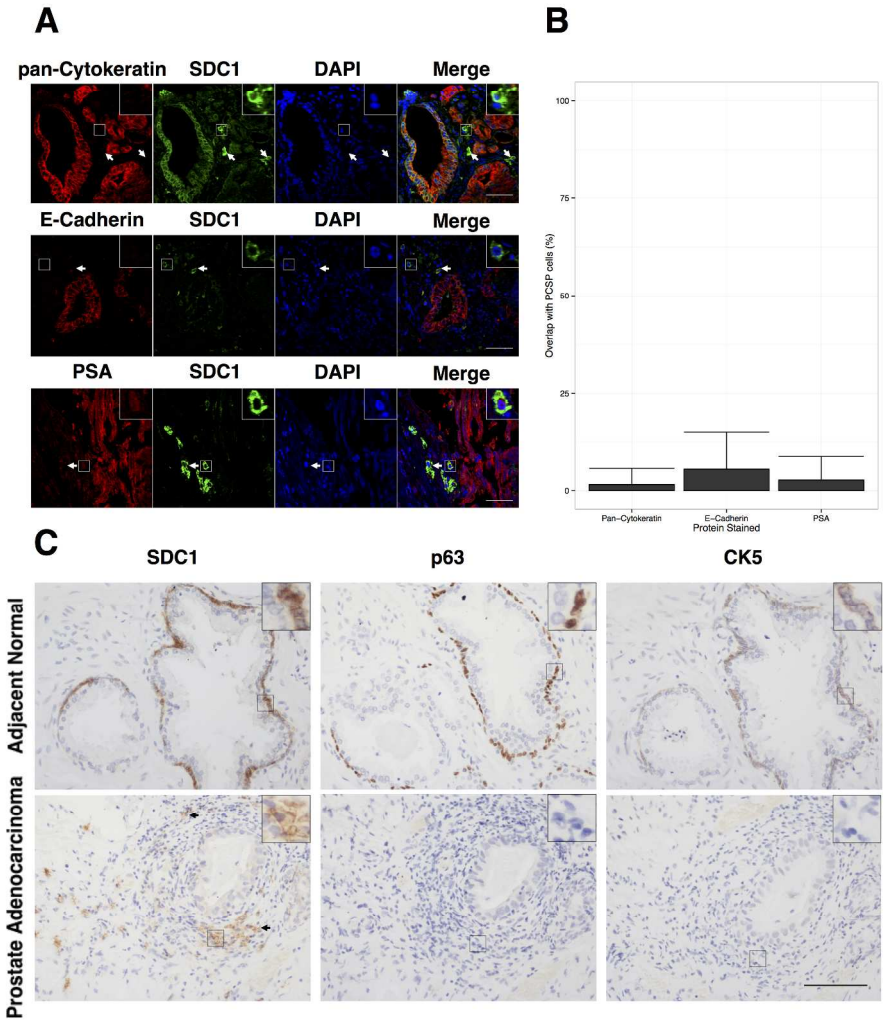

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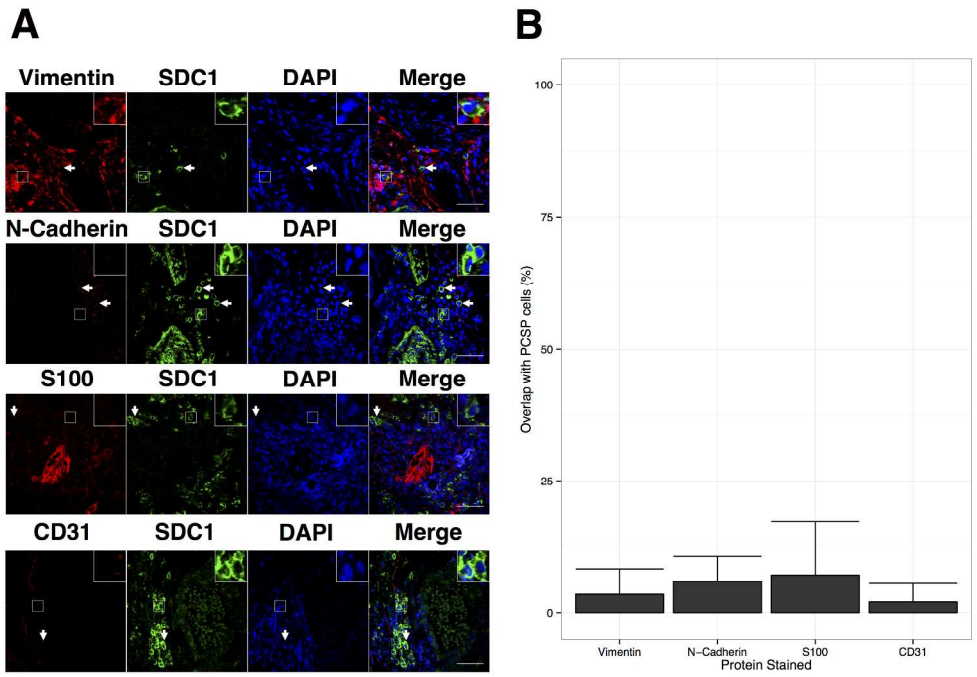

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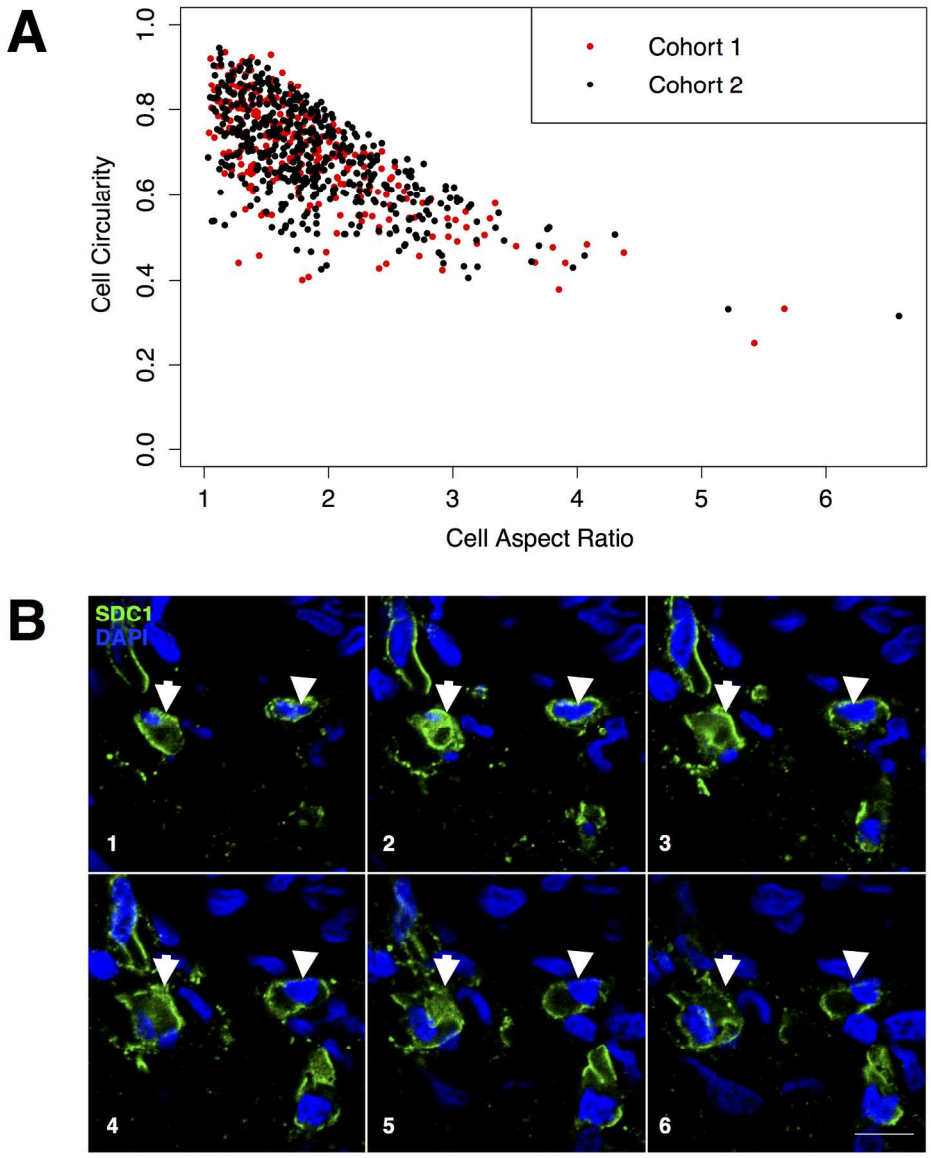

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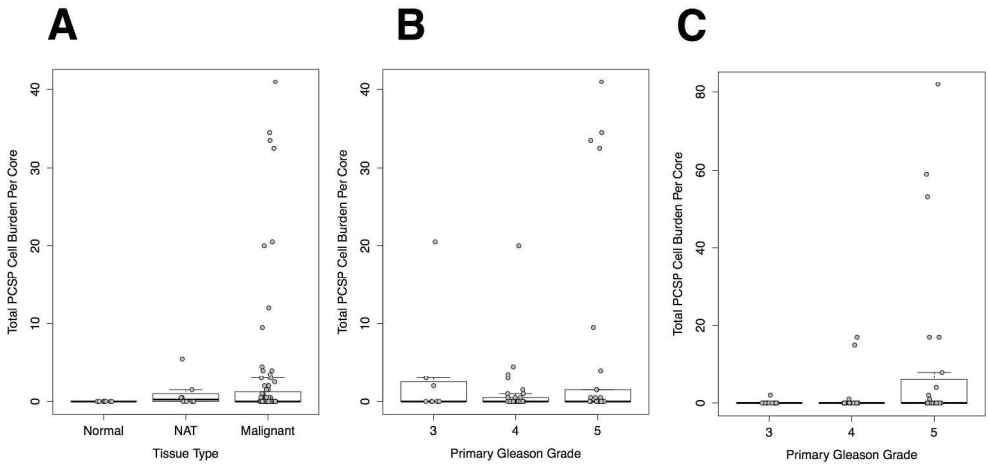

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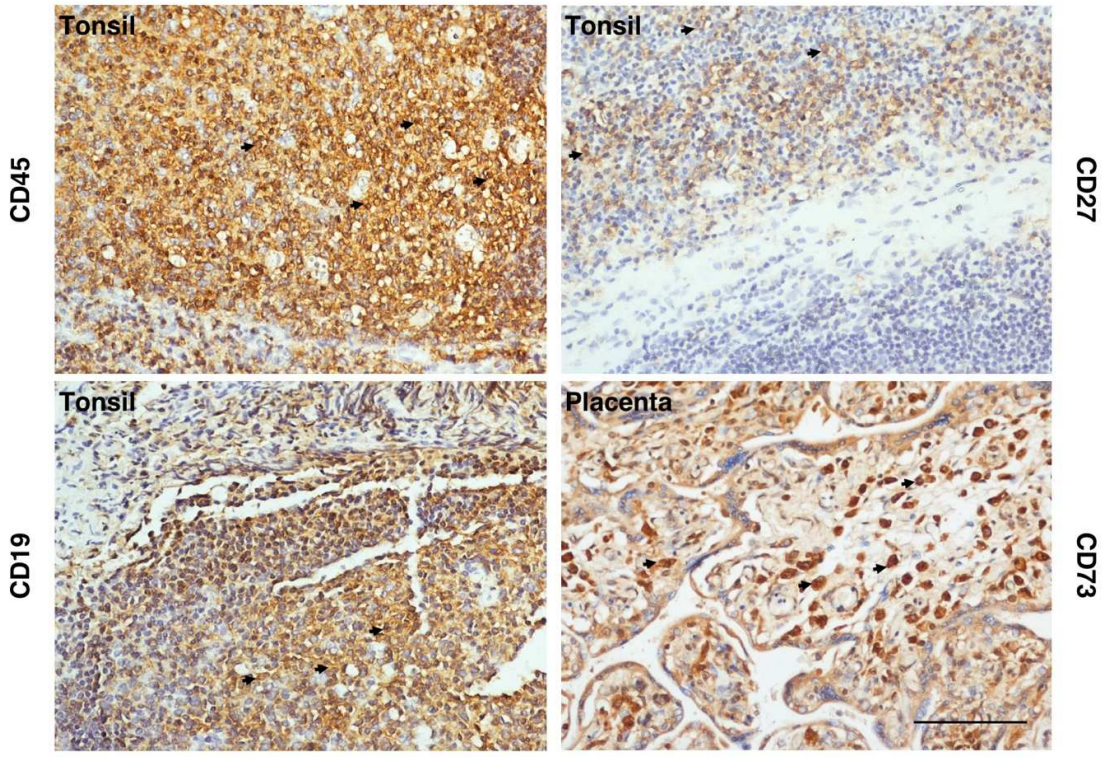

Supplementary Figure 1. Immunohistochemistry with anti-immune cell marker antibodies on positive control paraffin-embedded tissue sections. As expected, CD45, CD19, CD27 showed cytoplasmic and membranous staining in lymphocyte populations in the tonsil and CD73 marked lymphocytes in the placenta. Nuclei were counterstained with haematoxylin (blue). IHC scale bars – 100um.

	Cohort 1 (PR1921)	%	Cohort 2 (PR803b)	%
Number of Patients	80		71	
Age:				
Range	20-85		20-87	
Mean	67.85		67.6	
Median	69.5		70	
<50	2	2.5	3	4.2
50-59	5	6.3	4	5.6
60-69	33	41.3	27	38.0
>=70	40	50.0	37	52.1
Clinical Stage:				
I	6	7.5	2	2.8
II	37	46.3	33	46.5
III	14	17.5	10	14.1
IV	22	27.5	26	36.6
ND	1	1.3	0	0.0
Primary Gleason Grade:				
3	8	10.0	19	26.8
4	36	45.0	24	33.8
5	30	37.5	24	33.8
ND	6	7.5	4	5.6
Overall Gleason Grade:				
<=Gleason 3+4	8	10.0	16	22.5
>=Gleason 4+3	66	82.5	51	71.8
ND	6	7.5	4	5.6
T Category:				
T1	4	5.0	2	2.8
T2	47	58.8	40	56.3
T3	22	27.5	21	29.6
T4	6	7.5	8	11.3
ND	1	1.3	0	0.0
N Category:				
N0	64	80.0	51	71.8
N1	15	18.8	18	25.4
N2		0.0	2	2.8
ND	1	1.3	0	0.0

M Category				
M0	64	80.0	49	69.0
M1	15	18.8	22	31.0
ND	1	1.3	0	0.0
SDC1 Epithelial Reactivity:				
Absent	66	82.5	57	80.3
Present	14	17.5	13	18.3
ND	0	0.0	1	1.4
PCSP Cell Presence:				
Absent	52	65.0	56	78.9
Present	28	35.0	15	21.1

Antibody	Species of Origin	Clonality [Clone Number]	Working Dilution	Supplier	Catalogue No.
Syndecan-1 (SDC1)	Mouse	Monoclonal [B-A38]	1:200	Novus Biologicals	NB100-64980
Syndecan-1 (SDC1)	Rabbit	Polyclonal	1:50	Santa-Cruz Biotechnology	Sc-5632
Syndecan-1 (SDC1)	Mouse	Monoclonal [MI15]	1:25	Dako	N/A
CD45	Rabbit	Polyclonal	1:50	Santa-Cruz Biotechnology	Sc-25590
CD19	Rabbit	Monoclonal [EPR5906]	1:250	Abcam	Ab134114
CD27	Rabbit	Monoclonal [EPR8569]	1:100	Abcam	Ab131254
CD73	Rabbit	Polyclonal	1:25	Abcam	Ab115289
Pan-Cytokeratin	Mouse	Monoclonal [C-11]	1:100	Sigma-Aldrich	C2931
E-Cadherin	Mouse	Monoclonal [4A2C7]	1:25	Thermo Fisher Scientific	33-4000
Prostate-Specific Antigen (PSA)	Rabbit	Polyclonal	1:50	Dako	A0562
p63	Mouse	Monoclonal [BC4A4]	1:50	Abcam	Ab735
Cytokeratin -5	Rabbit	Polyclonal	1:1000	Abcam	Ab24647
Vimentin	Mouse	Monoclonal [V9]	1:50	Santa-Cruz Biotechnology	Sc-620

N-Cadherin	Rabbit	Polyclonal	1:100	Abcam	Ab18203
S100	Rabbit	Polyclonal	1:2000	Dako	Z0311
CD31	Mouse	Monoclonal [JC70A]	1:30	Dako	M0823

	Cohort 1 (PR1921)		Cohort 2 (PR803b)	
	Mean across samples	**Stdev across samples**	**Mean across samples**	**Stdev across samples**
Area	66.42	35.09	72.14	41.31
Circularity	0.69	0.12	0.70	0.13
Aspect Ratio	1.88	0.62	1.83	0.68
Roundness	0.58	0.16	0.60	0.17
Solidity	0.88	0.06	0.88	0.07